Assessment of RNA integrity in the postmortem pig colonic tissue ex vivo[1]

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ABSTRACT: Surgical removal of porcine intestinal tissue followed by an ex vivo challenge is an alternative technique of testing the anti-inflammatory effect of bioactive compounds in the intestine of live pigs. We investigated the effects of ex vivo incubation of porcine colonic tissue on the quantity and quality of total RNA over a 12-h time period. Colonic tissue of pig (n = 6) was surgically removed immediately postslaughter and incubated for 0, 3, 6, and 12 h in a humidified cell culture incubator with 5% CO_2 at 37°C. Tissue samples were processed for RNA extraction. The quantity and quality of total RNA were assessed on a NanoDrop Spectrophotometer and an Agilent 2100 Bioanalyzer, respectively. Ex vivo incubation had an effect on both the quantity ($P < 0.001$) and quality ($P < 0.001$) of total RNA. Relative to the RNA yield at 0 h (505.0 ± 48.64 μg/mg), the yield was significantly reduced after 6 h (227.6 ± 25.52 μg/mg; $P < 0.001$) and 12 h (159.3 ± 24.19 μg/mg; $P < 0.001$) of incubation. The 28S and 18S rRNA bands were visibly intact after 0, 3, and 6 h of incubation. However, after 12 h of incubation, a degraded RNA profile was evident. The RNA integrity number (RIN) values for the 0, 3, 6, and 12 h of incubation were 9.4 ± 0.10, 9.0 ± 0.10, 6.7 ± 0.17 ($P < 0.001$), and 3.3 ± 0.24 ($P < 0.001$), respectively. The transcript abundances of 4 constitutively expressed genes glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta actin (*ACTB*), beta 2-microglobulin (*B2M*), and peptidylprolyl isomerase A (*PPIA*) were reduced at both 6 and 12 h of incubation. It is concluded that ex vivo incubation of porcine colonic tissue up to 3 h postmortem generates good quality total RNA suitable for gene expression studies.

Key words: colon, gene expression, RNA integrity number value, RNA stability

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INTRODUCTION

The technique of ex vivo challenge of intestinal tissue has been used to investigate the immunomodulatory potential of bioactive food supplements in pigs (Smith et al., 2011; Leonard et al., 2012). The technique involves dissection of the intestinal tissue postmortem followed by ex vivo incubation with a pro-inflammation inducing agent, such as bacterial lipopolysaccharide. Prolonged incubation of the intestinal tissue ex vivo may adversely affect the abundance of gene transcripts. Hence, any deterioration of the RNA integrity in the postmortem tissue makes such tissue unsuitable for quantitative gene expression analysis (Denise, 2007).

The acquisition of high-quality intact RNA from the postmortem tissue is a fundamental requirement for quantitative evaluation of gene expression. Currently, there is no information available on the influence of incubation time ex vivo on the integrity of total RNA in porcine colon tissue. In this study, we investigated the effects of ex vivo incubation time on both the quantity and quality of total RNA in porcine colon and its subsequent effects on the gene expression of 4 constitutively expressed genes.

MATERIALS AND METHODS

Animals and Tissue Sample Collection and Treatment of Colon

All experimental procedures were conducted under experimental license from the Irish Department of Health in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendments of the Cruelty to Animals Act, 1876) Regulation, 1994.

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Colon samples were collected from pigs (n = 6) (Large White × Landrace; age 32 d) following euthanasia as outlined previously (Leonard et al., 2012). A section of approximately 1.5 × 1.5 cm of the colon was transferred into 1 mL Dulbecco's Modified Eagle Medium and incubated for 0, 3, 6, and 12 h in a humidified cell culture incubator with 5% CO_2 at 37°C. Tissue samples were collected in RNAlater (Ambion, Austin, TX) and processed for RNA extraction.

Extraction and Assessment of RNA Integrity and Quantitative Real-Time PCR Analysis

Total RNA was extracted from 25 mg tissue samples using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. To eliminate any genomic DNA contamination, RNA samples were subjected to deoxyribonuclease treatment and re-extracted using standard phenol-chloroform extraction method. Total RNA was finally dissolved in 50 µL 0.1% diethylpyrocarbonate-treated water and stored at –80º C.

The total RNA was quantified using a NanoDrop-ND 1000 (Thermo Fisher Scientific Inc., Boston, MA). An aliquot of total RNA (1 µL) was analyzed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) using RNA Nano LabChips (Caliper Technologies Corporation, Hopkinton, MA). The RNA integrity number (**RIN**) value is an empirical measure of RNA integrity based on the intensities of 28S and 18S rRNA bands.

Synthesis of cDNA was performed using RevertAid H minus First Strand cDNA synthesis kit (Fermentas, GmbH, St Leone-Rot, Germany) and oligo dT primers on 1 µg of total RNA according to the manufacturer's instructions. The expression of 4 constitutively expressed genes [glyceraldehyde 3-phosphate dehydrogenase (***GAPDH***), beta actin (***ACTB***), beta 2-microglobulin (***B2M***), and peptidylprolyl isomerase A (***PPIA***)] were measured and analyzed as previously described (Bahar et al., 2007).

Statistical Analysis

The RIN values, RNA yields, and fold change data were analyzed as a complete randomized design using the GLM procedure of SAS (SAS Institute, Cary, NC). All the data was checked initially for outliers and normality using the PROC Univariate procedure of SAS.

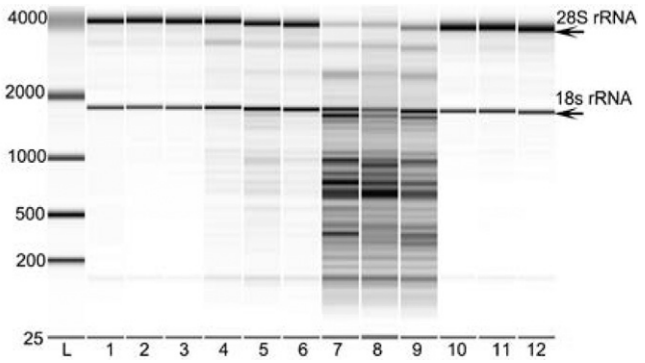

Figure 1. Assessment of RNA integrity (Agilent Bioanalyzer profile) in the postmortem colon of pig subjected to ex vivo incubation for 0 (lanes 7, 8, and 9), 3 (lanes 1, 2, and 3), 6 (lanes 10, 11, and 12), and 12 h (lanes 4, 5, and 6). Gel profile includes results from three random animals for each time point. Lane L: RNA ladder and 28S rRNA and 18S rRNA bands are marked with arrows.

RESULTS

Analysis of the Bioanalyzer profile of total RNA indicated that the 28S and 18S rRNA bands were visibly intact at 0, 3, and 6 h of incubation (Figure 1). However, after 12 h, these 2 rRNA populations disintegrated and a degraded RNA profile was apparent. Incubation time had a significant ($P < 0.001$) effect on the RIN value (Table 1). Incubation for 0, 3, 6, and 12 h gave RIN values of 9.4 ± 0.10, 9.0 ± 0.10, 6.7 ± 0.17, and 3.3 ± 0.24, respectively. Incubation time also had a significant effect ($P < 0.001$) on the yield of total RNA (Table 1). Whereas the RNA yield was at its maximum at the 0 h (505.0 ± 48.64 µg/mg), a reduction was evident after 3 h (367.2 ± 13.35 µg/mg), 6 h (227.6 ± 25.52 µg/mg; $P < 0.001$), and 12 h (159.3 ± 24.19 µg/mg; $P < 0.001$) of incubation.

The ex vivo incubation time significantly altered the relative quantities of the transcripts of *GAPDH* ($P < 0.001$), *ACTB* ($P < 0.001$), *B2M* ($P < 0.001$), and *PPIA* ($P < 0.001$) genes (Figure 2). Relative to the 0 h time point, whereas the relative abundances of *ACTB* and *B2M* gene transcripts were marginally reduced after 3 h, there was no reduction detected in the relative abundances of *GAPDH* and *PPIA* transcripts. In contrast, there were significant reductions in the abundance of all the 4 gene transcripts at both 6 and 12 h of incubation.

Table 1. Assessments of total RNA in the postmortem colon of pig (n = 6) subjected to ex vivo incubation for 0, 3, 6, and 12 h. Data presented as mean (±SE).

Parameters	Ex vivo incubation time, h			
	0	3	6	12
RNA yield, µg/mg	505.0 ± 48.64	367.2 ± 13.35	227.6 ± 25.52[a]	159.3 ± 24.19[a]
RNA integrity	9.4 ± 0.10	9.0 ± 0.10	6.7 ± 0.17[a]	3.3 ± 0.24[a]

[a]Mean different ($P < 0.001$) when compared with 0 h.

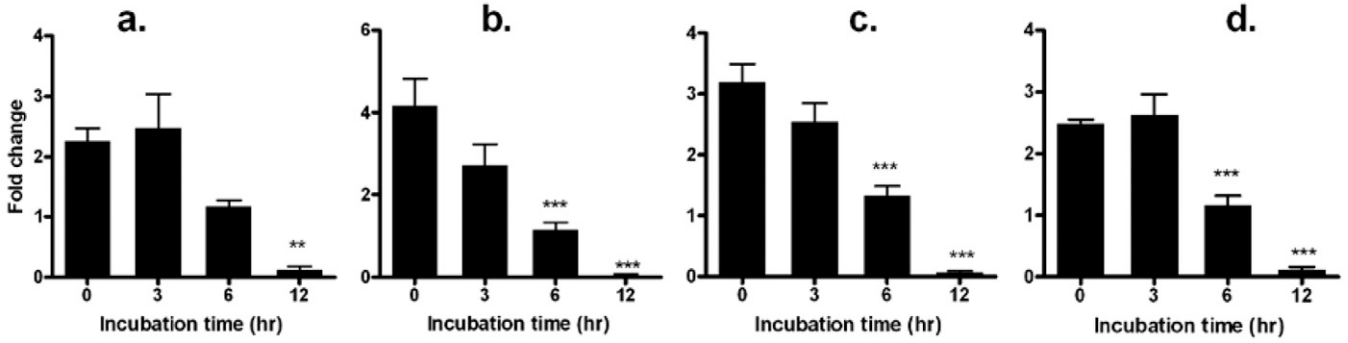

Figure 2. Relative abundance of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (a), beta actin (*ACTB*) (b), beta 2-microglobulin (*B2M*) (c), and peptidylprolyl isomerase A (*PPIA*) (d) gene transcripts in the postmortem colon of pig (n = 6) subjected to ex vivo treatment for 0, 3, 6, and 12 h. Data presented as mean (±SE).

DISCUSSION

This study investigated the integrity of total RNA and abundance of gene transcripts in colon of pigs in an ex vivo experimental setup. It is evident from our study that colonic tissue exposed to ex vivo incubation conditions for up to 3 h can generate intact RNA and gene transcripts comparable with that obtained from fresh tissue. However, tissue incubated for 6 h and 12 h had evidence of RNA degradation and lower transcript abundance.

Recovering highly intact RNA from the colonic tissue subject to postmortem treatment remains a challenge because high levels of ribonuclease activity in the intestinal tissue rapidly degrades RNA (Ibberson et al., 2009). In this experiment, highly intact RNA with RIN values >9.0 were obtained following a 3 h ex vivo incubation of the pig colonic tissue. In addition, there was no significant alteration in the transcript abundance of 4 constitutively expressed genes (*GAPDH*, *ACTB*, *B2M*, and *PPIA*) during this period indicating that the ex vivo treatment of porcine colon can be performed for up to 3 h without affecting integrity of the mRNA transcripts.

Colonic explants of human have previously been used as a tool to study host parasite interaction in an ex vivo experimental setup (Bansal et al., 2009). More recently, an ex vivo model was used as a means of studying the interactions of human parasites with the porcine colon (Girard-Misguich et al., 2012). Previously, we have successfully detected upregulation of pro-inflammatory cytokine gene expression in postmortem porcine colon following treatment with lipopolysaccharide ex vivo (Smith et al., 2011). The findings of this experiment demonstrate that harvesting the tissue within 3 h of ex vivo incubation is acceptable in terms of total RNA and transcript integrity.

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